Myristate can be used as a carbon and energy source for the asymbiotic growth of arbuscular mycorrhizal fungi

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Arbuscular mycorrhizal (AM) fungi, forming symbiotic associations with land plants, are obligate symbionts that cannot complete their natural life cycle without a host. The fatty acid auxotrophy of AM fungi is supported by recent studies showing that lipids synthesized by the host plants are transferred to the fungi, and that the latter lack genes encoding cytosolic fatty acid synthases. Therefore, to establish an asymbiotic cultivation system for AM fungi, we tried to identify the fatty acids that could promote biomass production. To determine whether AM fungi can grow on medium supplied with fatty acids or lipids under asymbiotic conditions, we tested eight saturated or unsaturated fatty acids (C12 to C18) and two β-monoacylglycerols. Only myristate (C14:0) led to an increase in the biomass of *Rhizophagus irregularis*, inducing extensive hyphal growth and formation of infection-competent secondary spores. However, such spores were smaller than those generated symbiotically. Furthermore, we demonstrated that *R. irregularis* can take up fatty acids in its branched hyphae and use myristate as a carbon and energy source. Myristate also promoted the growth of *Rhizophagus clarus* and *Gigaspora margarita*. Finally, mixtures of myristate and palmitate accelerated fungal growth and induced a substantial change in fatty acid composition of triacylglycerol compared with single myristate application, although palmitate was not used as a carbon source for cell wall biosynthesis in this culture system. Our findings demonstrate that myristate boosts the asymbiotic growth of AM fungi and can also serve as a carbon and energy source.

fatty acid auxotrophy | immobilized cell culture | mycorrhizal symbiosis | pure culture

Arbuscular mycorrhizal (AM) fungi belonging to the subphylum Glomeromycotina (1) form symbiotic associations with >70% of land plant species (2). AM fungi provide hosts with minerals taken up via hyphal networks in soil and in return receive carbon sources, such as sugars and lipids derived from plant photosynthates. This is considered an obligate symbiotic relationship, as AM fungi can complete their life cycle only through colonization of their host. Nevertheless, several studies of AM fungal culture without hosts have been published. One of these studies showed that the cocultivation of the AM fungus *Rhizophagus irregularis* (formerly *Glomus intraradices*) with bacterial strains of *Paenibacillus validus*, separated from each other by dialysis membranes, induced secondary spore formation in the AM fungus (3). Another study reported that some fatty acids, including palmitoleic acid and (*S*)-12-methyltetradecanoic acid (anteiso-C15:0), induced the formation of infection-competent secondary spores in asymbiotic cultures of AM fungi (4). These results suggest that AM fungi may be cultured independently from host plants under artificial conditions.

In nature, the life cycle of AM fungi proceeds as follows. Resting spores of AM fungi germinate, and then germ tubes emerge from the spores and elongate into the soil. After colonization into plant roots, AM fungi form highly branched hyphal structures, called arbuscules, in plant cortical cells, which are the sites of nutrient exchange between AM fungi and their hosts. After receiving carbon sources from their hosts, AM fungi activate the formation of extraradical hyphal networks in soil and form spores on their hyphae. Obtaining carbon sources from their hosts for the production of energy and the carbon skeleton of fungal cell components is a key step in AM fungal growth and reproduction. In particular, tracing experiments and NMR analyses have shown that hexoses are transferred to AM fungi as a carbon source (5–7). Moreover, several AM fungal monosaccharide transporters have been identified (8–10).

Recently, lipids have been identified as another plant-derived carbon source (11–15). Since no fatty acid biosynthesis in extraradical hyphae has ever been detected, lipids were assumed to be synthesized in intraradical hyphae and transferred to extraradical hyphae and spores (7, 16). However, AM fungal genomes do not possess genes encoding cytosolic fatty acid synthases involved in de novo fatty acid biosynthesis, indicating that AM fungi cannot produce long-chain fatty acids by themselves (17–20). On the other hand, during AM symbiosis, plants activate fatty acid biosynthesis and transfer lipids, presumably

Significance

The origins of arbuscular mycorrhizal (AM) fungi, which form symbiotic associations with land plants, date back >460 Mya. During evolution, these fungi acquired an obligate symbiotic lifestyle, and thus depend on their host for essential nutrients. In particular, fatty acids are regarded as crucial nutrients for the survival of AM fungi, owing to the absence of genes involved in de novo fatty acid biosynthesis in the AM fungal genomes that have been sequenced so far. Here we show that myristate initiates AM fungal growth under asymbiotic conditions. These findings will advance the pure culture of AM fungi.

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16:0 β-monoacylglycerol (β-MAG), to AM fungi via arbuscules (11–15). In fact, in plant mutants defective in fatty acid biosynthetic genes that are specific to the pathway supplying lipids to their symbionts, AM fungi cannot develop arbuscules, and their root colonization is reduced.

These findings indicate that AM fungi require exogenous fatty acids for their growth. Once AM fungi take up fatty acids, they can utilize them through fatty acid desaturases and elongases encoded by genes present in their genomes and expressed in intraradical hyphae (16–18, 20–23). Thus, we examined whether AM fungi can grow and produce fertile spores under asymbiotic conditions through the application of fatty acids.

Results

Myristate Activates AM Fungal Growth. Initially, we screened several fatty acids and β-MAGs to identify chemicals that can promote the

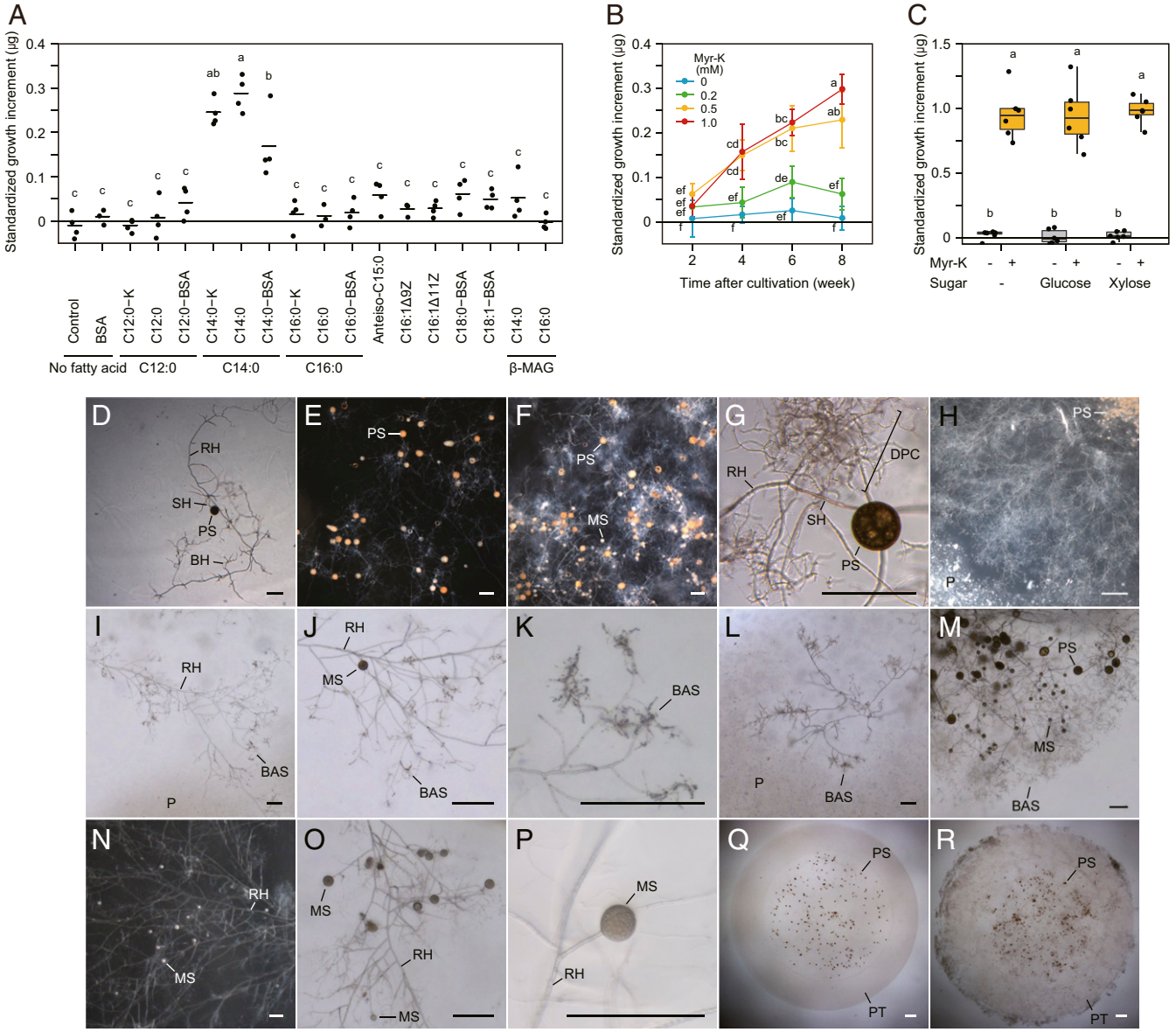

Fig. 1. Asymbiotic culture of *R. irregularis* in the presence of fatty acids. (*A*) Standardized growth increment (*Materials and Methods*) during 8 wk in the modified SC solid medium supplemented with potassium salts of fatty acids, fatty acids and β-MAGs, or fatty acid-BSA conjugates. Horizontal lines indicate mean values ($n = 3$ to 4). (*B*) Time course of biomass production with different amounts of Myr-K. Error bars represent 95% confidence intervals ($n = 5$ to 6). (*C*) Biomass production in an immobilized cell culture system. Immobilized fungal spores were incubated in half-strength modified SC medium supplemented with combinations of Myr-K and sugars after 8 wk of cultivation. The boxes show the first quartile, the median, and the third quartile; the whiskers reach to 1.5 times the interquartile range; and data points for each treatment are displayed ($n = 6$). The same lowercase letter indicates no significant difference (Tukey's test, $P < 0.05$; *A*–*C*). (*D*–*P*) Fungal growth in the solid medium without fatty acids (*D* and *E*) or with Myr-K (*F*–*P*). (*D*) Germinating spore. (*E* and *F*) Hyphal elongation without fatty acids (*E*) and with Myr-K (*F*) after 8 wk of cultivation. (*G*) DPC-like structures formed around a parent spore. (*H*) Radial growth of fungal mycelium. (*I*) Elongation of runner hyphae. (*J*) Branching of runner hyphae and formation of BAS. (*K*) Magnified image of BAS. (*L*) Front line of elongated mycelium. The medium around the fungal hyphae became transparent, indicating that the precipitates of metal soaps were solubilized. (*M*) Myristate-induced secondary spores generated around the parent spores. (*N* and *O*) Myristate-induced spores formed along the runner hyphae. (*P*) Magnified image of a myristate-induced spore. (*Q* and *R*) Fungal growth in the immobilized cell culture system without fatty acids (*Q*) and with Myr-K (*R*) after 8 wk of cultivation. Sample details are provided in *SI Appendix*, Table S2. BH, branching hypha; MS, myristate-induced spore; P, precipitate of metal soaps; PS, parent spore; PT, Phytagel tablet; RH, runner hypha; SH, subtending hypha. (Scale bars: 200 μm for *D*–*G* and *I*–*P* and 1,000 μm for *H*, *Q*, and *R*.)

growth of *R. irregularis* under asymbiotic conditions. These compounds were added to modified *Saccharomyces cerevisiae* synthetic complete (SC) medium (*SI Appendix*, Table S1) with different dissolution methods, including fatty acid salts in aqueous solution, fatty acids and β-MAGs dissolved in ethanol, and fatty acids conjugated with BSA. Fatty acid-BSA conjugates (C12:0, C14:0, C16:0, C18:0, and C18:1) were dissolved to a final concentration of 0.5 mM, whereas potassium salts of fatty acids (C12:0, C14:0, and C16:0) were converted into an insoluble form due to the formation of metal soap with Ca^{2+} and Mg^{2+} in SC medium. Moreover, fatty acids (C12:0, C14:0, C16:0, anteiso-C15:0, C16:1Δ9Z, and C16:1Δ11Z) and β-MAGs (C14:0 and C16:0) were aggregated in solid culture (*SI Appendix*, Fig. S1).

Symbiotically generated spores of *R. irregularis* were used as starting material (parent spores) for asymbiotic culture. In the absence of fatty acids, no increase in biomass was detected for *R. irregularis* (Fig. 1 *A* and *B*). After spore germination, each *R. irregularis* spore generated a thick, short subtending hypha that soon branched to produce straight-growing thick hyphae (hereinafter referred to as runner hyphae), with several thin lateral hyphae displaying a low level of ramification (Fig. 1 *D* and *E*). Hyphal elongation ceased within 1 or 2 wk after germination. In contrast, myristates (C14:0), unlike other tested chemicals, substantially increased fungal biomass regardless of the method used for their incorporation in the medium (Fig. 1*A*). Moreover, the increase in fungal biomass in the culture system over time depended on the amount of myristate (Fig. 1*B*). Consequently, the total dry weight of a 2-mo-old colony treated with 1 mM potassium myristate (Myr-K) was double that of parent spores (*SI Appendix*, Fig. S1*A*).

Surprisingly, 16:0 β-MAG and palmitate (16:0), candidate compounds released from arbusculated host cells during symbiosis (11, 15), did not increase biomass production. However, during cultivation with myristate, vigorous hyphal development and subsequent differentiation of secondary spores were observed (Fig. 1 *F–P* and *SI Appendix*, Fig. S1). After germination, *R. irregularis* differentiated a few densely packed coil (DPC)-like structures from the runner hyphae in the vicinity of the parent spore (Fig. 1*G*). The DPC is an extensively branched hyphal structure that was first observed in *R. irregularis* cocultivated with *P. validus* (24) and whose existence was confirmed in fungal materials supplemented with palmitoleic acid and anteiso-C15:0 (4). Furthermore, *R. irregularis* elongated its runner hyphae by generating short-branched hyphae similar to branched absorbing structures (BAS) (25) and expanded its habitat by generating new runner hyphae (Fig. 1 *H–J*). The branched hyphal structures (hereinafter referred to as BAS), small bunches of short, thin branches, were generated from the runner hyphae at short intervals (Fig. 1*K*).

Interestingly, at the start of cultivation with a Myr-K supplement, numerous precipitates of putative myristate salt were observed throughout the growth medium; however, the precipitates around actively growing hyphae were progressively solubilized (Fig. 1 *H*, *I*, and *L*). Moreover, myristate-induced secondary spores were frequently observed along the runner hyphae in the vicinity of parent spores (Fig. 1*M*). Myristate-induced spores also occurred apically or intercalarily along the lateral branches of the extensively growing runner hyphae (Fig. 1 *N* and *O*). These spores were ∼50 μm in diameter, almost one-half the size of parent spores (Fig. 1 *F*, *M*, and *P*). In the presence of palmitoleic acid (C16:1Δ9Z) in the medium, extensive hyphal branching and secondary spore formation were observed, consistent with the results reported by Kameoka et al. (4). However, in our system, hyphal growth was not associated with an increase in biomass by the application of palmitoleic acid (Fig. 1*A* and *SI Appendix*, Fig. S1*E*). When lauric acid (C12:0) was applied as a lauric acid-BSA conjugate, *R. irregularis* showed active elongation of runner hyphae with few DPC, BAS, or secondary spores (*SI Appendix*, Fig. S1*F*); however, this conjugate did not significantly increase fungal biomass (Fig. 1*A*). Thus, because the lauric acid-BSA conjugate was effective on hyphal elongation even at low concentrations (1 and 10 μM), lauric acid is not likely to be used as a macronutrient for *R. irregularis* (*SI Appendix*, Fig. S1*H*).

We further developed our culture system to promote fungal growth under asymbiotic conditions. First, we examined fungal growth in liquid culture with a modified SC medium containing Myr-K. Myristate was effective in enhancing fungal biomass in the liquid culture (*SI Appendix*, Fig. S2*A*). After germination, precipitates of metal soaps attached to the hyphal surface (*SI Appendix*, Fig. S2*B*). As the fungal hyphae continued to elongate and became tangled, the metal soaps on the surface gradually disappeared. However, typical BAS were not recognized due to the aggregation of fungal hyphae, although highly branched fine hyphae were observed in the presence of myristate (*SI Appendix*, Fig. S2*C*). In contrast to solid culture, very few secondary spores formed in liquid culture.

Next, we tested an immobilized cell culture system in which an inoculum of *R. irregularis* spores embedded in the center of a Phytagel tablet was incubated in modified SC liquid medium (*SI Appendix*, Fig. S3*A*). This culture system can prevent the aggregation of fungal hyphae and facilitate the diffusion of medium components in the Phytagel tablets. Notably, AM fungal growth in the immobilized cell culture system with Myr-K was increased by threefold to fourfold compared with that in solid and liquid cultures (Fig. 1 *A* and *C* and *SI Appendix*, Fig. S2*A*). Hyphal elongation was very active, and some hyphae even spread out of the Phytagel tablets and continued to grow (Fig. 1 *Q* and *R* and *SI Appendix*, Fig. S3 *B–D*). The elongation pattern of hyphae was similar to that in solid culture; for instance, DPC-like structures, runner hyphae, BAS, and myristate-induced spores were observed (*SI Appendix*, Fig. S3 *E–I*). In addition, myristate-induced spores were still smaller than parent spores (*SI Appendix*, Fig. S3*J*).

Because monosaccharides are utilized by AM fungi as carbon sources during symbiosis (5–7), we examined whether monosaccharides influenced the growth of *R. irregularis* in an immobilized cell culture system. In the absence of Myr-K, neither glucose nor xylose promoted biomass production (Fig. 1*C*). Similarly, no additional biomass increase was detected on treatment with a combination of monosaccharides and Myr-K. The modified SC medium contained 1 mM glycerol, which is a potential carbon source because *R. irregularis* can absorb and metabolize it under asymbiotic conditions (26). However, glycerol did not increase biomass production of *R. irregularis* with or without myristate (*SI Appendix*, Fig. S1 *B–D*).

To ascertain whether myristate exerts similar effects in other AM fungal species, *Rhizophagus clarus* (order Glomerales, family Glomeraceae) and *Gigaspora margarita* (order Diversisporales, family Gigasporaceae), belonging to the same genus as and a different order than *R. irregularis*, respectively, were cultured in the same conditions. When incubated in a medium without fatty acids, *R. clarus* produced short runner hyphae from the parent spores and formed several small secondary spores, although hyphal elongation soon ceased (*SI Appendix*, Fig. S4). Conversely, in the presence of myristate, *R. clarus* showed active hyphal growth and sporulation, accompanied by an increase in biomass. After germination, runner hyphae were vigorously elongated and sometimes branched dichotomously. In addition, BAS-like structures were generated at regular intervals from several long runner hyphae. Myristate-induced secondary spores were also formed on short lateral branches deriving from runner hyphae. Similar to those seen in *R. irregularis*, the myristate-induced spores

of *R. clarus* were one-half the size of the parent spores and had thinner spore walls.

Because we could not obtain an adequate number of sterile spores of *G. margarita*, we only analyzed its growth pattern. *G. margarita* produced longer hyphae than *R. irregularis* and *R. clarus* even in the absence of myristate, possibly owing to its large spores with more abundant energy reserves (*SI Appendix*, Fig. S5). After transfer to a myristate-containing medium, *G. margarita* produced much longer runner hyphae, from which BAS-like structures were generated. No newly formed spores were observed in *G. margarita*, although auxiliary cells (subglobose, hyaline cells clustered in groups of approximately 10 with spiny ornamentations borne on coiled hyphae) differentiated in both media.

Myristate-Induced Spores Have Infection Capability. Myristate-induced spores of *R. irregularis* began to differentiate at 2 wk after cultivation in solid medium. The number of myristate-induced spores increased with time and the amount of myristate and finally reached 1.4 spores per parent spore by 8 wk after treatment (Fig. 2*A*). However, these spores were still one-half the size of the parent spores (Fig. 2*B*). The number of *R. irregularis* myristate-induced spores in the immobilized cell culture system was equal to that in solid culture, and the addition of xylose further promoted their production, which reached up to 2.5 spores per parent spore (Fig. 2*C* and *SI Appendix*, Fig. S6). Myristate-induced spores were initially white to pale yellow and gradually turned to a yellow-brown color, similar to symbiotically generated spores (*SI Appendix*, Figs. S6*A* and S7*A*). Moreover, both spores induced by myristate and those produced symbiotically showed three spore wall layers originating from cylindrical subtending hyphae, although wall layers of the myristate-induced spores were thinner than those of the symbiotically generated spores (*SI Appendix*, Fig. S7*B*). Numerous nuclei, vacuoles, and lipid droplets were observed in both spore types (*SI Appendix*, Fig. S7 *A* and *B*).

To test whether myristate-induced spores could colonize plant roots, single spores generated in the immobilized cell culture system with Myr-K and xylose were inoculated to carrot hairy roots. A total of 245 spores were examined in six independent experiments (*SI Appendix*, Table S3). The myristate-induced spores displayed infectivity toward hairy roots, triggering the production of next-generation mature spores on the extraradical hyphae that emerged from the roots (Fig. 2 *D–G*). Approximately one-half of the germinated spores could colonize hairy roots and produce daughter spores, although large variations in the germination rate and infectivity of spores were observed among the trials due to the effect of experimental manipulations (*SI Appendix*, Table S3).

AM Fungi Utilize Myristate as a Carbon and Energy Source. To address the use of fatty acids by AM fungi under asymbiotic conditions, we analyzed fatty acid uptake using two fluorescent fatty acid derivatives of different chain lengths: 4,4-difluoro-5-methyl -4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoic acid (C_1-BODIPY 500/510 C_{12}) and 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-hexadecanoic acid (BODIPY FL C_{16}). *R. irregularis* absorbed C_1-BODIPY 500/510 C_{12} through its BAS (not runner hyphae) within 10 min of initial exposure (Fig. 3*A*). The probe was first taken up from the hyphal tips of BAS (Fig. 3*A*), then fluorescent signals were observed over time in lipid body-like structures (27, 28) within runner hyphae, which were translocated by cytoplasmic streaming (*SI Appendix*, Fig. S8*B* and Movie S1). BODIPY FL C_{16} was also absorbed by the BAS within 4 h, while faint probe signals were observed during a 10-min period after the exposure (*SI Appendix*, Fig. S9 *A–C*). Long exposure to these probes resulted in signals localized in myristate-induced spores as well as in the BAS, runner hyphae, and DPC, but parent spores were rarely labeled (*SI Appendix*, Figs. S8 *A–E* and S9 *D–H*). Moreover, germ tubes deriving from germinating spores incubated in sterilized water also took up the fluorescently labeled fatty acids, indicating that the activation of

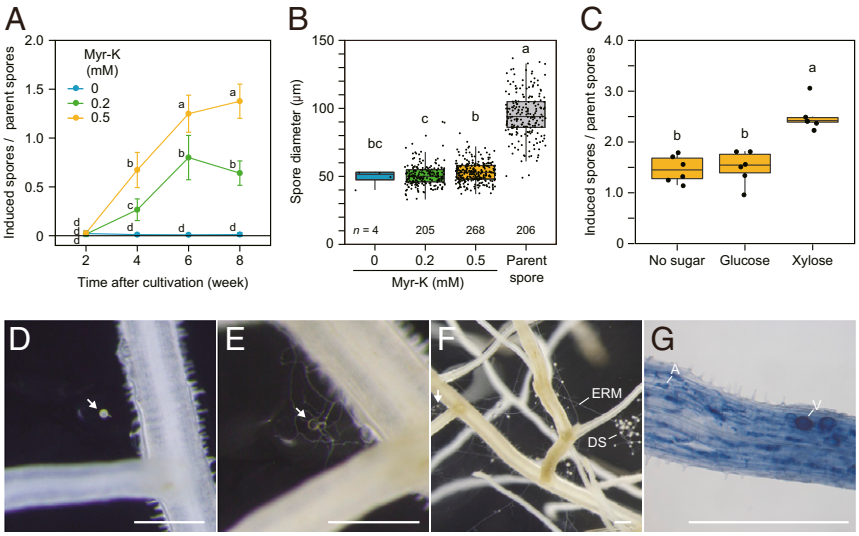

Fig. 2. Spore formation of *R. irregularis* under asymbiotic conditions. (*A* and *B*) Number (*A*) and diameter (*B*) of myristate-induced spores generated in solid medium containing different amounts of Myr-K. Spore diameter was measured after 8 wk of cultivation. (*C*) Number of myristate-induced spores in an immobilized cell culture containing 0.5 mM Myr-K and 5 mM monosaccharides after 8 wk of cultivation. Error bars in *A* represent 95% confidence intervals. For each boxplot, the boxes show the first quartile, the median, and the third quartile; the whiskers reach to 1.5 times the interquartile range; and data points for each treatment are displayed. The same lowercase letter indicates no significant difference. In *A*, data were transformed as $\log_{10}(x + 0.5)$; $P < 0.05$, Tukey's test; $n = 3$ to 6. In *B*, $P < 0.05$, Steel–Dwass test; in *C*, $P < 0.05$, Tukey's test, $n = 6$. (*D–G*) Inoculation of a single myristate-induced spore to carrot hairy roots. (*D*) Inoculated spore produced in the immobilized cell culture containing Myr-K and xylose. (*E*) Germination of the inoculated spore. (*F*) Daughter spores (DS) on extraradical mycelia (ERM) emerged from the carrot hairy roots. (*G*) Colonization of the carrot hairy roots by *R. irregularis*. Arrows indicate an inoculated myristate-induced spore. A, arbuscule; V, vesicle. Sample details are provided in *SI Appendix*, Table S2. (Scale bars: 500 μm.)

AM fungal hyphae by myristate is not necessarily required for fatty acid uptake (*SI Appendix*, Figs. S8 *F–G* and S9 *I–J*). Gene expression analysis confirmed that Myr-K transcriptionally activated β-oxidation, the glyoxylate cycle, gluconeogenesis, and the TCA cycle by 3 h after its application (Fig. 3*B*). Interestingly, a gene encoding a *N*-myristoyl transferase (NMT), which catalyzes the myristoylation of proteins, was up-regulated by myristate.

To further assess whether myristate provides the carbon skeleton for fungal cell components, we applied [1-^{13}C]myristate to the culture medium and analyzed the cell wall components of *R. irregularis*. After the extraction of fungal cell walls, chitin and chitosan were converted into glucosamine (GlcN) through acid hydrolysis. Liquid chromatography-mass spectrometry (LC-MS) analysis of the extracted GlcN demonstrated significantly higher relative ion intensity of M + 1 in AM fungi supplemented with [1-^{13}C]myristate compared with AM fungi supplemented with nonlabeled myristate (mean, 16.4% vs. 6.1%) (Fig. 3*C*). This finding indicates that external myristate was taken up by

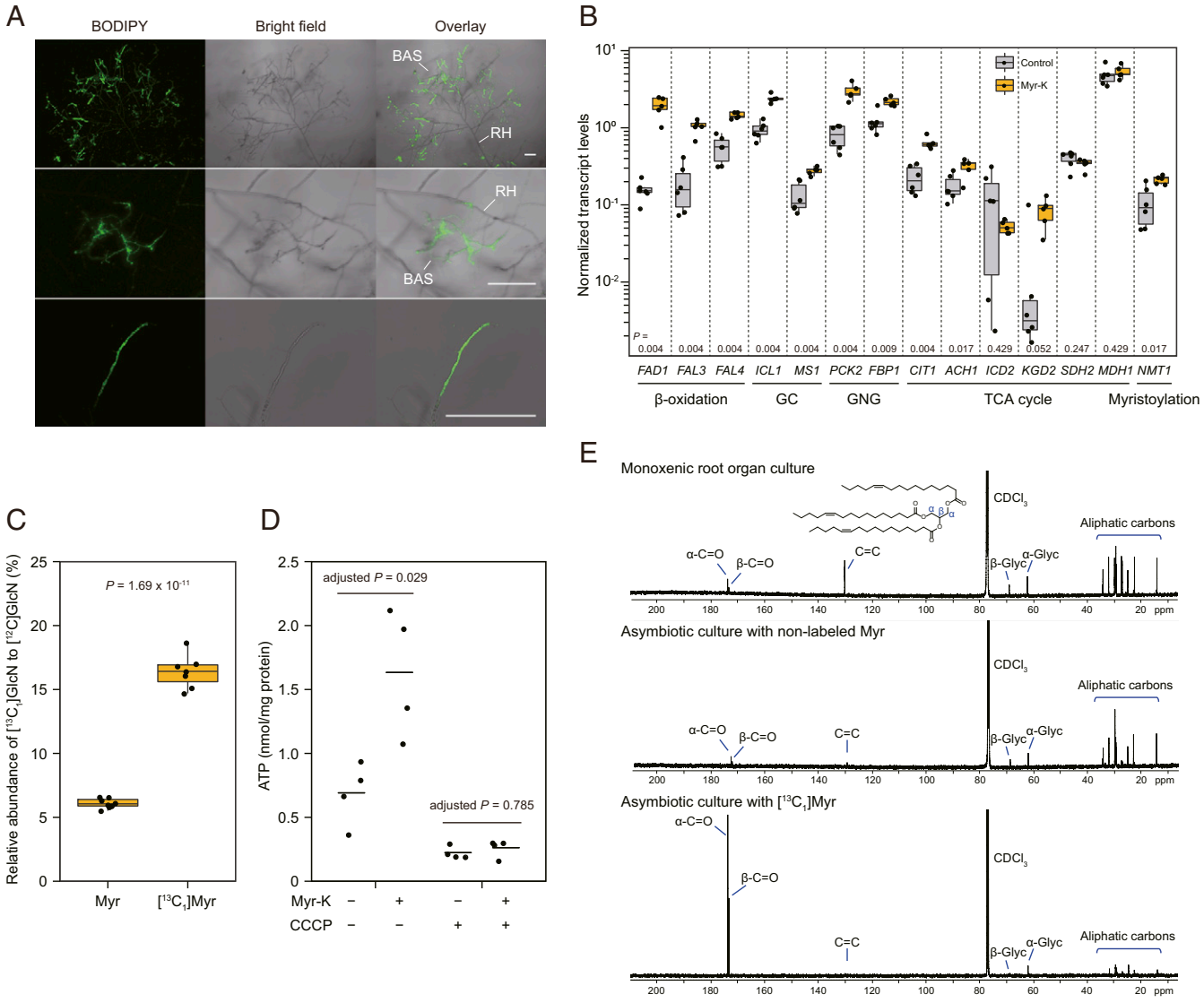

Fig. 3. Utilization of fatty acids by *R. irregularis* under asymbiotic conditions. (*A*) Uptake of the fluorescently labeled fatty acid derivative C_1-BODIPY 500/510 C_{12}. Shown are a cluster of BAS (*Upper*), magnified BAS (*Middle*), and a single BAS hypha (*Lower*). Optical sections captured using a confocal laser scanning microscope are projected. Fluorescence images and superimposed bright field images are shown. RH, runner hypha. (Scale bars: 200 μm.) (*B*) Gene expression analysis in *R. irregularis* at 3 h after the application of Myr-K or water (control) shown by real-time RT-PCR. The *R. irregularis* elongation factor 1 beta and actin genes were used for normalization. For each boxplot, the boxes show the first quartile, the median, and the third quartile; the whiskers reach to 1.5 times the interquartile range; and data points for each treatment are displayed (Myr-K, $n = 5$; control, $n = 6$). *P* values were calculated using the Wilcoxon–Mann–Whitney *U* test. Details of genes are provided in *SI Appendix*, Table S4. GC, glyoxylate cycle; GNG, gluconeogenesis. (*C*) Incorporation of carbon derived from exogenous myristate into cell wall components of *R. irregularis*. AM fungi were grown in the immobilized cell culture system and supplemented with 0.5 mM neutralized myristic acid (Myr) or [1-$^{13}C_1$]myristic acid ([$^{13}C_1$]Myr) for 8 wk. Glucosamine (GlcN) was extracted from the cell walls of the fungal materials without parent spores. The relative abundance of [$^{13}C_1$]GlcN ([M + 1 + H]$^+$; *m/z* 181.19) to [^{12}C]GlcN ([M + H]$^+$; *m/z* 180.19) was calculated by LC-MS analysis. The reported *P* values are based on Student's *t* test (Myr: $n = 8$; [$^{13}C_1$]Myr: $n = 7$). (*D*) ATP content in *R. irregularis* at 12 h after the application of Myr-K or distilled water (control) in the presence or absence of CCCP. Horizontal lines indicate the mean values ($n = 4$). *P* values are based on Student's *t* test with Bonferroni correction. (*E*) ^{13}C-NMR spectra of TAG isolated from mycelial extracts of *R. irregularis* grown in monoxenic root organ culture (*Upper*), asymbiotic culture supplemented with 1 mM neutralized myristic acid (*Middle*), or [1-$^{13}C_1$]myristic acid (*Lower*). (*Inset*) Chemical structure of TAG. Experiments were repeated twice independently, with similar results.

R. irregularis and utilized for the biosynthesis of chitin and chitosan in fungal cell walls.

In addition, to evaluate the use of myristate as an energy source, we measured adenosine triphosphate (ATP) production after the application of myristate. In particular, we applied myristate to germinating hyphae and measured their ATP content at 12 h after application. Notably, ATP content increased by 2.4-fold in the presence of myristate (Fig. 3*D*). In addition, when carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), an inhibitor of mitochondrial membrane depolarization, was simultaneously applied to the hyphae, ATP content did not increase even after fatty acid application.

The translocation of the fluorescent fatty acid probes to myristate-induced spores (*SI Appendix*, Figs. S8 and S9) prompted us to examine whether myristate is utilized to synthesize the major storage lipid, triacylglycerol (TAG). To this purpose, we cultured *R. irregularis* in medium supplemented with [1-^{13}C]myristic acid. After extraction of lipids from the fungal materials, TAG was purified through preparative thin layer chromatography and analyzed by ^{13}C-NMR. In the spectrum, two peaks at 173.1 and 173.4 ppm, corresponding to the carboxyl carbons of acyl chains at the α and β positions, respectively, were observed at a much higher intensity than that seen in the spectrum of TAG prepared from nonlabeled fungal materials or monoxenically cultured *R. irregularis* (Fig. 3*E*). This finding shows that exogenous myristate was incorporated into TAG, especially in its acyl carboxyl components.

Since no signals denoting the presence of unsaturated TAG fatty acids in myristate-fed AM fungi were detected by ^{13}C-NMR (Fig. 3*E*), the fatty acid composition is likely different from that in symbiotically generated spores. Furthermore, AM fungi are predicted to obtain C16:0 β-MAG or palmitic acid from the host under symbiotic conditions (11, 15). Analysis of the growth and lipid composition of AM fungi in the presence of a combination of myristate and C16:0 β-MAG or palmitate revealed that palmitate enhanced hyphal growth and biomass production of *R. irregularis* when combined with 0.5 mM myristate, but C16:0 β-MAG exerted no significant effect (Fig. 4*A* and *SI Appendix*, Fig. S10). Secondary spore formation was also stimulated by the combination of myristate and palmitate (Fig. 4*B*). In the presence of palmitate or C16:0 β-MAG together with myristate, spores displayed similar morphology but a slightly larger spore size compared with those induced by myristate (Fig. 4*C* and *SI Appendix*, Figs. S7 and S10). In addition, lipid droplets extracted from spores incubated in myristate seemed to be in a solid state, whereas those induced by a mixture of myristate and palmitate were liquid, similar to symbiotically generated spores (*SI Appendix*, Fig. S7*A*). This observation may reflect a change in the lipid composition of TAG.

To prove this hypothesis, we analyzed the fatty acid composition (the major acyl group C14:0, C16:0, and C16:1Δ11) of AM fungal TAG by gas chromatography-mass spectrometry (GC-MS). In myristate-fed AM fungi, C14:0 was the dominant acyl group, while C16:0 and C16:1Δ11 were found in trace amounts (Fig. 4*D*). Furthermore, [1-$^{13}C_1$]myristate-labeling experiments showed that exogenous myristate was directly incorporated into TAG as C14:0 acyl groups (Fig. 4*E*). A significant fraction of C16:0 and C16:1Δ11 also derived from [1-$^{13}C_1$]myristate, indicating that myristate taken up into the fungus was elongated to C16:0 and desaturated into C16:1Δ11. In contrast, TAG in fungal materials supplemented with both myristate and palmitate contained high amounts of C16:0 and C16:1Δ11 (Fig. 4*D*). The majority of these acyl groups are derived from the exogenous [1-$^{13}C_1$]palmitate (Fig. 4*E*). C16:1Δ11, a signature fatty acid for most AM fungi except those of the genera *Gigaspora*, *Archaeospora*, and *Paraglomus* (29), is likely generated from palmitate by the desaturase DES2, also known as OLE1-like, that is constitutively expressed in AM fungal hyphae (23, 30, 31).

We next analyzed fungal cell wall components after incubation in a mixture of myristate and palmitate. The relative ion intensity of [$^{13}C_1$]GlcN from AM fungi supplemented with [1-$^{13}C_1$]palmitate and nonlabeled myristate was similar to the ion intensity of a nonlabeled fatty acid mixture, indicating that carbon from exogenous palmitate was not incorporated into chitin and chitosan of fungal cell walls (Fig. 4*F*). Conversely, myristate-derived carbon was used for cell wall biosynthesis when [1-$^{13}C_1$]myristate and nonlabeled palmitate were supplied.

Discussion

AM fungi have an obligate biotrophic lifestyle; that is, they depend on host-derived nutrients for their growth. Recently, AM fungi have been shown to receive lipids from the host via arbuscules (11–15); however, how these fungi utilize these lipids as nutrients is largely unknown. Here we show that myristate (C14:0) can serve as a carbon and energy source for the hyphal growth of *R. irregularis* under asymbiotic conditions, thereby providing evidence of increasing AM fungal biomass in a pure culture system. Myristate also promoted the growth of *R. clarus* and *G. margarita*, suggesting that myristate is effective in promoting asymbiotic growth in a wider range of AM fungal species. *R. irregularis* and *R. clarus* elongated their hyphae and formed secondary spores in a similar manner. In contrast, no secondary spores were observed in *G. margarita*, although long runner hyphae with BAS were generated in the presence of myristate. Considerable variation in the fatty acid composition of spores was observed between the genera *Rhizophagus* and *Gigaspora* (16, 29), and thus their utilization of and response to exogenous fatty acids also may differ.

Notably, we confirmed that myristate-induced spores of *R. irregularis* are infective propagules capable of generating symbiotic daughter spores, as was previously demonstrated for palmitoleic acid-induced secondary spores (4). Moreover, the observation that fluorescently labeled fatty acid derivatives of different chain length accumulated in *R. irregularis* hyphae suggested that AM fungi are likely to absorb fatty acids nonspecifically. However, myristate was the sole fatty acid effective in promoting biomass production in our culture conditions. This finding is surprising given that 16:0 β-MAG or its related chemicals are thought to be transferred from the host to AM fungi under symbiotic conditions (11, 15). Meanwhile, because the plant enzymes FatM and RAM2, which are responsible for AM-specific lipid biosynthesis, have been found to use C14:0-containing molecular species as well as C16:0 as a substrate in vitro (14, 15), and because myristate and C14:0 α-MAG have been detected in mycorrhizal roots and fungal spores, albeit in very small amounts (11, 16, 29), it is plausible that myristate is provided to AM fungi by the host.

Myristate may be essential for the biological processes of AM fungi via myristate-specific metabolic pathways. Indeed, myristate is used for the lipid modification of proteins, a process known as protein *N*-myristoylation, in a variety of eukaryotes (32–34). Protein *N*-myristoylation is catalyzed by NMT, which transfers myristate from myristoyl-CoA to the N-terminal glycine residue of target proteins (35). *N*-myristoylated proteins are involved in diverse cellular processes, such as protein phosphorylation and signal transduction. Interestingly, disruption of the *NMT* gene causes recessive lethality in several fungal species (36–38). As indicated by the up-regulation of the *R. irregularis NMT1* gene on application of myristate, myristate may contribute to AM fungal growth by inducing protein *N*-myristoylation, as well as by acting as a carbon source. If this were indeed the

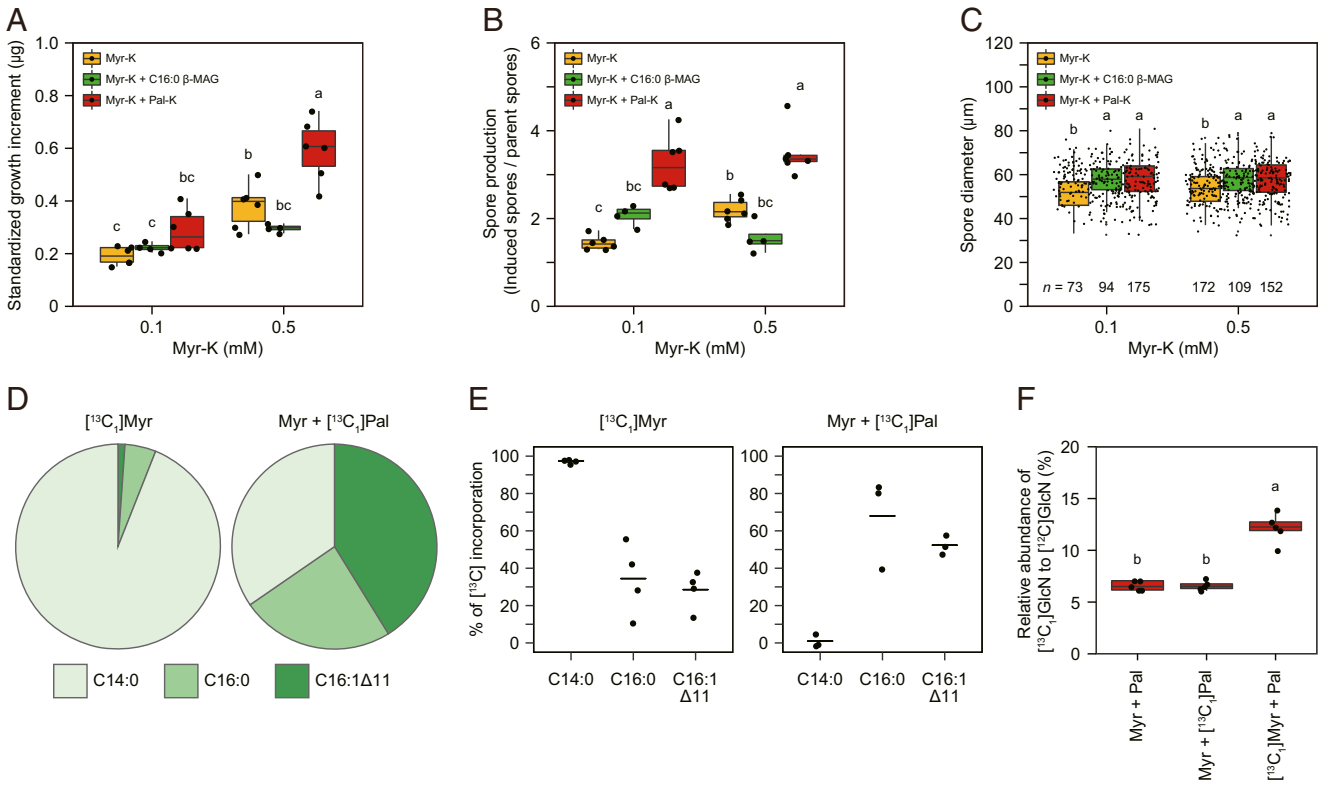

Fig. 4. Effects of fatty acid mixtures on the growth and sporulation of *R. irregularis*. AM fungi were cultured in the immobilized cell culture system and supplemented with Myr-K (0.1 or 0.5 mM), either alone or in combination with 0.5 mM C16:0 *sn*-2 monoacylglycerols (β-MAG C16:0) or potassium palmitate (Pal-K) ($n = 4$ to 6). *SI Appendix*, Table S2 provides sample details. Shown are the standardized growth increment (*A*) and number (*B*) and diameter (*C*) of myristate-induced secondary spores after 8 wk of cultivation. For each boxplot, the boxes show the first quartile, the median, and the third quartile; the whiskers reach to 1.5 times the interquartile range; and data points for each treatment are displayed. The same lowercase letter indicates that there is no significant difference ($P < 0.05$, Tukey's test,). (*D*) Composition of C14:0, C16:0, and C16:1Δ11 fatty acids of TAG isolated from fungal materials grown in asymbiotic culture and supplemented with 0.5 mM neutralized [1-$^{13}C_1$]myristic acid ([$^{13}C_1$]Myr) or 0.1 mM Myr-K plus 0.5 mM neutralized [1-$^{13}C_1$]palmitic acid (Myr + [$^{13}C_1$]Pal). (*E*) Percentage of [^{13}C], derived from the labeled myristate or palmitate, incorporated into TAG. Horizontal lines indicate mean values ($n = 3$ to 4). (*F*) Incorporation of carbon derived from exogenous myristate and palmitate into cell wall components of *R. irregularis*. AM fungi were supplemented with labeled or nonlabeled neutralized myristic acid (0.5 mM) and palmitic acid (0.5 mM). GlcN was extracted from fungal cell walls without parent spores. Relative abundance of [$^{13}C_1$]GlcN ([M + 1 + H]$^+$, *m/z* 181.19) to [^{12}C]GlcN ([M + H]$^+$, *m/z* 180.19) was calculated using data from LC-MS analysis. The same lowercase letter indicates no significant difference (Tukey's test, $P < 0.05$, $n = 5$).

case, then we predict that fatty acids other than myristate would also be used for fungal growth if sufficient myristate to induce *N*-myristoylation were provided to AM fungi. Consistently, a mixture of myristate and palmitate further promoted fungal growth and led to marked increases in the proportions of C16:0 and C16:1Δ11 acyl groups in fungal TAG, which are the predominant groups in symbiotically grown *R. irregularis* (17, 39). However, carbon derived from exogenous palmitate was not used for cell wall biosynthesis. On the other hand, in the presence of myristate, palmitate might stimulate the metabolism of stored lipids in parent spores for asymbiotic growth or serve as an energy source. The former hypothesis is consistent with the results of our [1-$^{13}C_1$]palmitate labeling experiment, in which exogenous [1-$^{13}C_1$]palmitate and nonlabeled palmitate, likely derived from stored lipids, were incorporated into lipids of newly produced hyphae and secondary spores. However, palmitate might become available as a carbon source for AM fungal growth under both symbiotic and asymbiotic conditions in the presence of factors that were not considered in our culture system. Future studies may better elucidate how AM fungi use different types of fatty acids.

Fungal cell proliferation requires a sufficient supply of nutrients. In particular, carbon sources are critical for producing the building blocks of cells and generating ATP. The observed incorporation of ^{13}C in TAG and cell wall components of *R. irregularis* when [1-^{13}C]myristate was supplied indicates that myristate is utilized as a carbon source to synthesize cellular components. During symbiosis, β-oxidation, the glyoxylate cycle, and gluconeogenesis are active in AM fungi, and these metabolic pathways have been proposed to play a crucial role in the generation of carbohydrates from lipids (7, 26, 40, 41). Since the expression of major genes involved in β-oxidation, the glyoxylate cycle, gluconeogenesis, and the TCA cycle was induced by the application of myristate, absorbed myristate is likely metabolized through these metabolic pathways, and the resulting carbohydrates are used to build the backbone of fungal cells. Furthermore, the increased ATP levels on the addition of myristate indicates that myristate also can serve as an electron donor for the respiration of AM fungi.

In our culture systems, hexoses did not induce an increase in fungal biomass, although xylose in combination with myristate triggered the production of a great number of secondary spores. This finding implies that AM fungi can hardly use sugars as carbon sources under these conditions. This observation is consistent with the results of previous ^{13}C-labeling experiments showing reduced hexose uptake by germinating spores compared with intraradical hyphae (40). However, hexoses derived from the host are taken up via fungal monosaccharide transporters in

intraradical hyphae and/or arbuscules during symbiosis (6). In addition, the monosaccharide transporter *MST2* gene is expressed even in BAS formed on the medium in a carrot hairy root system (23) and induced in extraradical hyphae treated with xylose (9). Thus, we cannot rule out the possibility that AM fungi can utilize exogenous sugars for their growth under asymbiotic conditions.

A characteristic response of AM fungi to myristate is the branching of runner hyphae and the formation of BAS. To date, a number of chemical compounds, including strigolactones, 2-hydroxy fatty acids, palmitoleic acid, and branched fatty acids, have been found to induce hyphal branching during the presymbiotic phase (4, 42, 43). For example, strigolactones stimulate fifth- to sixth-order hyphal branching in *G. margarita* at extremely low concentrations (42) but induce only moderate hyphal branching in *Rhizophagus* sp. LPA8 (44). Moreover, two 2-hydroxy fatty acids, 2OH-C14:0 and 2OH-C12:0, also affect presymbiotic hyphal growth in *Gigaspora* spp., and these AM fungi were found to produce multiple lateral branches along the primary germ tubes; however, 2OH-C14:0 and 2OH-C12:0 do not elicit any morphological change in *R. irregularis* (43). In contrast, palmitoleic acid and branched fatty acids stimulate hyphal branching of *R. irregularis* and *R. clarus* at low concentrations but do not have this effect in *G. margarita* (4). In particular, palmitoleic acid induces high-degree short branching and sporulation. In the present study, however, the effect of myristate on hyphal branching and elongation was completely different from the effects of these known stimulants. Indeed, myristate induced extensive hyphal branching in *R. irregularis*, *R. clarus*, and *G. margarita* at high concentrations. In contrast, it is known that these AM fungi do not respond to low concentrations of myristate (4, 43). To explain this phenomenon, it is likely that myristate is utilized as a nutrient, with subsequent activation of fungal metabolism and gene expression, which results in the differentiation of BAS, DPC, and branched runner hyphae to further absorb myristate. Indeed, we observed that fatty acids were taken up by the fungal BAS and DPC. Then absorbed fatty acids or their metabolites are likely stored in lipid bodies and translocated to runner hyphae, as previously observed in symbiotic extraradical hyphae (26, 28, 41). Part of the fatty acids or lipids also might be delivered to newly formed spores, where they may be used for spore germination and subsequent hyphal elongation.

In conclusion, asymbiotic growth of AM fungi can be supported by externally supplied fatty acids, leading to the possibility of generating a pure culture of biotrophic AM fungi. Although myristate initiates AM fungal growth and sporulation, the size of myristate-induced spores remains small compared with that of symbiotically generated spores. Similar results were obtained for palmitoleic acid-induced spores (4). As smaller spores show low germination rates and infectivity (45), spore maturation in the absence of the host represents an exciting future challenge for the pure culture of AM fungi. Taken together, our findings provide further insight into the cellular and molecular biology of AM fungi and have important implications for the development of new strategies for the genetic transformation and production of inocula of these organisms.

Materials and Methods

More detailed information on the materials and methods used in this study is provided in *SI Appendix, Extended Methods*.

Biological Materials. Sterile spore suspensions of the AM fungus *R. irregularis* DAOM197198 (or DAOM181602, another voucher number for the same fungus) were purchased from Premier Tech. Hyphae included in the spore suspension were removed by density-gradient centrifugation using gastrografin, as described in *SI Appendix, Extended Methods*. *R. clarus* HR1 (MAFF520076) and *G. margarita* K-1 (MAFF520052) were also used for asymbiotic culture.

Asymbiotic Culture. Approximately 300 to 400 parent spores of *R. irregularis* were placed on 0.3% Phytagel (Sigma-Aldrich) plates containing modified SC medium (*SI Appendix*, Table S1) and then covered with 0.3% Phytagel dissolved in 3 mM magnesium sulfate in a 12-well culture plate. For liquid culture, Phytagel was removed from the medium. Then fatty acid salts, fatty acids in an organic solvent, or fatty acids conjugated with BSA were added to the medium, and the plates were incubated at 28 °C in the dark. Hyphal elongation was observed under a dissecting microscope and a light microscope.

Immobilized Cell Culture. An overview of the immobilized cell culture system is provided in *SI Appendix*, Fig. S3*A*. Here 6-mm-high, 17.5-mm-wide Phytagel tablets, with 3-mm-deep and 11.5-mm-wide circular incisions containing *R. irregularis* spores were transferred into a 6-well culture plate. Each well was filled with 5 mL of full- or half-strength modified SC liquid medium with an appropriate amount of fatty acids and monosaccharides. AM fungi were grown at 28 °C in the dark. During the culture period, the liquid medium was changed monthly.

Asymbiotic Culture of *R. clarus* and *G. margarita*. Sterile spores of *R. clarus* were prepared using a monoxenic system with carrot hairy roots. *G. margarita* spores were extracted from soil in pot culture and sterilized using chloramine T. These AM fungi were cultured in Phytagel covered with half-strength modified SC medium containing 0.5 mM Myr-K at 28 °C for 12 wk.

Measurement of Fungal Biomass. Fungal materials were recovered from gels in the wells of culture plates by melting the gels in citrate buffer and then weighed with a micro analytical balance. The number of parent spores in each well was counted in advance under a dissecting microscope. The standardized growth increment of AM fungi was calculated by dividing the total fungal dry weight in each well by the number of parent spores and then subtracting the mean dry weight of a parent spore.

Spore Morphology. Spores were mounted with polyvinyl alcohol-lactic acid-glycerol or Melzer's reagent for microscopic observation. Spores were incubated with 10 μM SYTO 13 Green Fluorescent Nucleic Acid Stain (Thermo Fisher Scientific) for 2 h and observed by epifluorescence microscopy. Transmission electron microscopy was performed to analyze the ultrastructure of spores as described by Kameoka et al. (4).

Single Spore Inoculation. A single myristate-induced spore produced in the immobilized cell culture system in half-strength modified SC medium supplemented with 0.5 mM Myr-K and 5 mM xylose was placed onto plates with carrot hairy roots using a pipette. The production of daughter spores on extraradical hyphae emerging from hairy roots was observed under a dissecting microscope. AM fungal colonization was confirmed by trypan blue staining.

Fatty Acid Uptake. *R. irregularis* was grown in an immobilized cell culture system with modified SC medium containing 0.5 mM Myr-K for 6 to 8 wk. Fungal hyphae were stained with 0.5 mM C_1-BODIPY 500/510 C_{12} or BODIPY FL C_{16} (Thermo Fisher Scientific) in modified SC medium. After a 10-min or 4-h incubation, fungal hyphae protruding outside a Phytagel tablet were observed using a laser scanning confocal microscope or an epifluorescence microscope. For the samples incubated for >1 d in medium containing the fluorescent probes, a Phytagel tablet containing fungal materials was melted by adding citrate buffer. Fluorescent signals were observed under an epifluorescence microscope. We also assayed fatty acid uptake by germ tubes grown in the absence of myristate.

LC-MS Analysis of Glucosamine. *R. irregularis* was incubated for 8 wk in half-strength modified SC medium with one of the following supplements: 0.5 mM nonlabeled myristic acid, 0.5 mM [1-$^{13}C_1$]myristic acid (Taiyo Nippon Sanso), 0.5 mM myristic acid and 0.5 mM palmitic acid, 0.5 mM myristic acid and 0.5 mM [1-$^{13}C_1$]palmitic acid (Taiyo Nippon Sanso), or 0.5 mM [1-$^{13}C_1$] myristic acid and 0.5 mM palmitic acid. All fatty acids used were neutralized in 200 mM potassium hydroxide. Extraction of GlcN from fungal biomass and LC-MS analysis are described in *SI Appendix, Extended Methods*. The relative

intensities of the molecular ion peaks of GlcN ($[M + H]^+$, *m/z* 180.19 and $[M + 1 + H]^+$, *m/z* 181.19) were monitored. The relative fraction of M + 1 with respect to that of M + 0 in the GlcN standard solution was 6.8%.

^{13}C-NMR Analysis of TAG. *R. irregularis* was cultured in modified SC solid medium supplied with 1 mM neutralized [1-^{13}C]myristic acid (Cambridge Isotope Laboratories) for 2.5 mo. Extraction of lipids from fungal biomass, purification of TAG, and ^{13}C-NMR analysis are described in *SI Appendix, Extended Methods*.

GC-MS Analysis of TAG. *R. irregularis* was incubated for 8 wk in half-strength modified SC medium with one of the following supplements: 0.5 mM neutralized myristic acid, 0.5 mM neutralized [1-$^{13}C_1$]myristic acid, 0.1 mM Myr-K and 0.5 mM neutralized palmitic acid, or 0.1 mM Myr-K and 0.5 mM neutralized [1-$^{13}C_1$]palmitic acid. The extraction of lipids from fungal biomass, purification of TAG, and GC-MS analysis are described in *SI Appendix, Extended Methods*.

Determination of ATP Content. *R. irregularis* spores were incubated in sterilized water at 28 °C for 5 d. Myr-K was added to the germinating spores at a final concentration of 0.5 mM. For the control, the protonophore CCCP was added simultaneously at a final concentration of 50 μM. After incubation for 12 h, fungal materials were crushed in PBS (pH 7.4) using a bead crusher (μT-12; Taitec). ATP concentration was measured using the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega). Protein concentration was assayed using the Qubit Protein Assay Kit (Thermo Fisher Scientific). ATP content in the germinating spores was calculated in nmol mg^{-1} of protein.

Quantitative RT-PCR. *R. irregularis* was grown in an immobilized cell culture system with modified SC medium containing 0.5 mM Myr-K for 3 wk. Subsequently, AM fungi were incubated in the absence of fatty acids for 11 d to induce fatty acid starvation. After starvation, Myr-K at a final concentration of 0.5 mM or sterilized water was added to the samples. After a 3-h incubation, fungal hyphae protruding outside a Phytagel tablet were recovered using forceps. RNA extraction, purification, cDNA synthesis, and semiquantitative PCR were conducted as described in *SI Appendix, Extended Methods*.

Statistical Analysis. All statistical analyses were performed using R version 3.5.2. Levene's tests were applied to check for heteroscedasticity between treatment groups. Data were transformed as $\log_{10}(x + 0.5)$ when necessary. To examine the differences among experimental groups, data were analyzed with Student's *t* test, Tukey's honest significant difference test, the Wilcoxon–Mann–Whitney *U* test, and the Steel–Dwass test, as appropriate. Differences at $P < 0.05$ were considered significant.

Data Availability. All data used in the study are included in the main text and *SI Appendix*. All protocols are described in *Materials and Methods* and *SI Appendix, Extended Methods* or in cited references. Any additional information desired is available on request from the corresponding author.

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